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Flagellin Is Required for Host Cell Invasion and Normal *Salmonella* Pathogenicity Island 1 Expression by *Salmonella enterica* Serovar Paratyphi A

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Salmonella enterica **serovar Paratyphi A is a human-specific serovar that, together with** ***Salmonella enterica*** **serovar Typhi and** ***Salmonella enterica*** **serovar Sendai, causes enteric fever. Unlike the nontyphoidal** ***Salmonella enterica*** **serovar Typhimurium, the genomes of *S.* Typhi and *S.* Paratyphi A are characterized by inactivation of multiple genes, including in the flagellum-chemotaxis pathway. Here, we explored the motility phenotype of *S.* Paratyphi A and the role of flagellin in key virulence-associated phenotypes. Motility studies established that the human-adapted typhoidal *S.* Typhi, *S.* Paratyphi A, and *S.* Sendai are all noticeably less motile than *S.* Typhimurium, and comparative transcriptome sequencing (RNA-Seq) showed that in *S.* Paratyphi A, the entire motility-chemotaxis regulon is expressed at significantly lowers levels than in *S.* Typhimurium. Nevertheless, *S.* Paratyphi A, like *S.* Typhimurium, requires a functional flagellum for epithelial cell invasion and macrophage uptake, probably in a motility-independent mechanism. In contrast, flagella were found to be dispensable for host cell adhesion. Moreover, we demonstrate that in *S.* Paratyphi A, but not in *S.* Typhimurium, the lack of flagellin results in increased transcription of the flagellar and the** ***Salmonella*** **pathogenicity island 1 (SPI-1) regulons in a FliZ-dependent manner and in oversecretion of SPI-1 effectors via type three secretion system 1. Collectively, these results suggest a novel regulatory linkage between flagellin and SPI-1 in *S.* Paratyphi A that does not occur in *S.* Typhimurium and demonstrate curious distinctions in motility and the expression of the flagellum-chemotaxis regulon between these clinically relevant pathogens.**

Salmonella enterica is one of the most prevalent human and animal pathogens, consisting of more than 2,500 serovars. Some *Salmonella* serovars, such as *Salmonella enterica* serovar Typhimurium, are ubiquitous pathogens that can infect a broad range of animal and human hosts. In contrast, other *Salmonella* serovars are host restricted and highly adapted in their pathogenesis. *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi A, and *Salmonella enterica* serovar Sendai are human-restricted serovars and the causative agents of enteric fever (1, 2). This is an invasive, life-threatening systemic disease with a global annual estimation of over 25 million cases, resulting in more than 200,000 deaths (3). In recent years, the occurrence of infection with *S.* Paratyphi A has been rising, and in some regions of the world (particularly in eastern and southern Asia), it is accountable for up to 50% of all enteric fever cases (4, 5). Increasing rates of infection, the lack of a commercially available vaccine, and steadily increasing resistance of *S.* Paratyphi A isolates to antimicrobial agents make *S.* Paratyphi A infections a significant public health concern.

Bacterial invasion of nonphagocytic cells is one of the hallmarks of *S. enterica* and is pivotal for its pathogenicity. Active invasion by *Salmonella* of eukaryotic cells is mediated by a type three secretion system (T3SS) encoded on *Salmonella* pathogenicity island (SPI) 1 (T3SS-1), which injects an array of translocated effectors directly into the host cell cytoplasm, enabling *Salmonella* engulfment and transport through the intestinal barrier (6).

Most of the *S. enterica* serovars are motile and harbor peritrichous flagella located at random points around the cell surface. The major structural protein that forms the flagellar filaments is flagellin. *S.* Typhimurium can express two antigenically distinct flagellin monomers, FliC and FljB, which are coordinately expressed by a phase variation mechanism mediated by the DNA invertase Hin, ensuring that only one gene is expressed at a given time (7). Various chemical stimuli received by receptors influence the rotation of the flagellum motor and allow *Salmonella* to respond by motility toward attractants and away from repellents (8). In addition to motility, flagella have been shown to participate in other microbial processes, including host cell adherence, biofilm formation, protein secretion, and host cell invasion (9).

Genomic comparison between *S.* Paratyphi A and *S.* Typhimurium has demonstrated a higher proportion of inactivated genes (pseudogenes) in *S.* Paratyphi A (4.1%) than in *S.* Typhimurium (1%), including in the flagellum-chemotaxis regulon (10). At least four genes involved in motility and chemotaxis, *fliB*, *tar*, *trg*, and *hin*, are inactivated in *S.* Paratyphi A but are intact in the *S.* Typhimurium genome. The frameshift mutation in *hin* prevents

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flagellin monomer switching and renders *S.* Paratyphi A monophasic for phase 1 flagella (FliC).

Considering the role of motility in bacterial pathogenesis (11) and the distinct clinical manifestations of typhoidal versus nontyphoidal serovars, we sought to study the motility phenotypes of *S.* Paratyphi A and other typhoidal serovars in comparison to *S.* Typhimurium and explored the role of flagellin in key virulence-associated phenotypes of *S.* Paratyphi A. Our results demonstrate that the human-specific typhoidal *S.* Typhi, *S.* Paratyphi A, and *S.* Sendai are noticeably less motile than *S.* Typhimurium and that the entire motility-chemotaxis regulon is expressed at significantly lower levels in *S.* Paratyphi A than in *S.* Typhimurium. Furthermore, we demonstrate that in *S.* Paratyphi A, the lack of phase I flagellin, FliC, results in upregulation of SPI-1 genes in a FliZ-dependent manner and in enhanced secretion of SPI-1 effectors. These results present a novel regulatory linkage between SPI-1 and flagellin that exists in *S.* Paratyphi A but not in *S.* Typhimurium and reveal unexpected expression and phenotypic differences in their motility.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains utilized in this study are listed in Table S1 in the supplemental material. Bacterial cultures were routinely maintained at 37°C in Luria-Bertani (LB) medium (BD Difco) supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 25 μg/ml chloramphenicol when appropriate.

Cloning and mutant construction. In-frame gene deletions were constructed with a λ-Red-mediated recombination system (12). The genes *fliC*, *fljB*, and *flhA* from *S.* Typhimurium SL1344 and *S.* Paratyphi A 45157 were cloned, together with their native regulatory regions, into the low-copy-number vector pWSK29. C-terminal two-hemagglutinin (2HA)-tagged versions of FliC and SopB were constructed within pWSK29, and a 2HA-tagged version of PrgJ was constructed in pACYC184. The primers used in this study are listed in Table S2 in the supplemental material.

Tissue cultures. The human colonic adenocarcinoma Caco-2 cell line was grown in Dulbecco's modified Eagle medium (DMEM)–F-12 medium supplemented with 20% fetal bovine serum (FBS) and 2 mM L-glutamine. The murine macrophage-like J774.1A cells were cultured in high-glucose (4.5 g/liter) DMEM supplemented with 10% heat-inactivated FBS, 1 mM pyruvate, and 2 mM L-glutamine. The human myelogenous leukemia THP-1 cell line was cultured in RPMI 1640 supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and 20% FBS. The mature macrophage-like state was induced by treating THP-1 cells for 24 h with phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml. Plastic-adherent cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) and incubated with fresh RPMI 1640 lacking PMA for 48 h. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Adhesion and invasion assays. Epithelial cells and macrophages were seeded at 5×10^4 and 2.5×10^5 cells/ml, respectively, in a 24-well tissue culture dish 18 h prior to bacterial infection. Experiments were carried out using the gentamicin protection assay as previously described (13). Caco-2 cells were infected at a multiplicity of infection (MOI) of ~1:50 with stationary-phase *Salmonella* cultures grown under microaerophilic conditions (15-ml tubes with no shaking). Macrophages were infected at an MOI of ~1:10 using stationary-phase cultures grown overnight. At the desired time points postinfection (p.i.), the cells were washed three times with PBS and harvested by addition of lysis buffer (0.1% SDS, 1% Triton X-100 in PBS). Appropriate dilutions were plated on LB plates for bacterial enumeration by CFU counting. *Salmonella* invasion was determined by the number of intracellular *Salmonella* cells at 2 h p.i. divided by the number of infecting bacteria. Adhesion was investigated by using 1 μg/ml cytochalasin D (CD). *Salmonella* adhesion was determined by the number of adherent *Salmonella* cells at 30 min p.i. divided by the number of infecting bacteria.

Western blot analysis. Bacterial pellets from cultures grown under microaerophilic conditions were resuspended in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Boiled samples were separated on 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were probed with anti-2HA tag (α-2HA tag) antibody (Abcam; ab18181, diluted 1:1,000) or anti-DnaK antibody (Abcam; ab69617, diluted 1:10,000). Goat α-mouse (Abcam; ab6721, diluted 1:10,000) conjugated to horseradish peroxidase was used as a secondary antibody, followed by detection with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia).

Transcriptome sequencing (RNA-Seq) and bioinformatics. Total RNA was extracted from two independent replicates of *S.* Typhimurium SL1344 and *S.* Paratyphi A 45157 grown to late log phase (optical density at 600 nm [OD_{600}], ~1.2) using the Qiagen RNAprotect Bacteria Reagent and the RNeasy minikit. rRNA was subtracted from 5 μg total RNA using the Ribo-Zero rRNA removal kit (Gram-negative bacteria; Illumina) following the manufacturer's recommendations. One hundred nanograms of the subtracted RNA was used as input to make a sequencing library using the TruSeq RNA sample preparation kit v2 (Illumina) following the manufacturer's recommendations. The RNA libraries were sequenced on an Illumina HiSeq 2500 in the paired-end mode with a read length of 100 bp at the University of California Irvine (Irvine, CA) (UCI) Genomics High-Throughput Facility. A total of ~153 million paired-end reads were obtained across the four RNA libraries, 99 to 99.5% of which, depending on the samples, were non-rRNA reads. Adaptor removal and quality trimming (Phred score, ≤20) of the raw reads were done using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). The quality trimmed reads were aligned to the respective reference genomes using Bowtie2 (version 2.0.6) (14). PCR and optical duplicates were removed from the alignments using MarkDuplicates within Picard tools (http://broadinstitute.github.io/picard/). Read counts within annotated features were generated using featureCounts (15). Genes differentially expressed between the two serovars were identified using edgeR (16).

Analysis of secreted proteins. Overnight cultures were diluted 1:100 in LB medium and grew for about 5.5 h to an OD_{600} of 2.4 to 2.6. Five milliliters of OD-normalized cultures were centrifuged at 13,000 rpm for 5 min, and the supernatant was filtered through a 0.22-μm syringe filter. Prechilled trichloroacetic acid (TCA) was added to a final concentration of 10%, placed on ice overnight, and then centrifuged at 13,000 rpm for 45 min at 4°C. The pellets were washed with acetone, allowed to dry, and dissolved in SDS sample buffer. Protein samples were boiled for 5 min, and equal volumes of 25 μl were separated on 12% polyacrylamide gels using SDS-PAGE. Bands were visualized by staining with 0.05% Coomassie brilliant blue.

Mass spectrometry analysis. Tryptic peptides were desalted using C_{18} tips (Harvard), dried, and resuspended in 0.1% formic acid. The resulting tryptic peptides from the supernatant were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS-MS) using a Q Exactive Plus mass spectrometer (Thermo) fitted with a capillary high-performance liquid chromatography (HPLC) instrument (easy nLC 1000; Thermo). The peptides were loaded onto a C_{18} trap column (0.3 by 5 mm; LC-Packings) connected online to a homemade capillary column (25 cm; 75-μm inside diameter [i.d.]) packed with Reprosil C18-Aqua (Dr Maisch GmbH; Germany) in solvent A (0.1% formic acid in water). The peptide mixture was resolved with a linear gradient (5 to 28%) of solvent B (95% acetonitrile with 0.1% formic acid) for 180 min, followed by a 5-min gradient of 28 to 95% and 25 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 μl/min. Mass spectrometry was performed in a positive mode (*m/z* 350 to 1,800) using a repetitive full MS scan followed by high-collision-induced dissociation (HCD) (at 35% of the normalized collision energy) of the 10 most dominant ions (>1 charge) selected from the first MS scan. A dynamic exclusion list was enabled with an exclusion duration of 20 s. The mass spectrometry data were analyzed and quantified using MaxQuant software 1.5 (http://www.maxquant.org) for peak picking identification and quantitation

using the Andromeda search engine, searching against the *S.* Paratyphi A or *S.* Typhimurium section of the UniProt database, with a mass tolerance of 20 ppm for the precursor masses and 20 ppm for the fragment ions. The minimal peptide length was set to 6 amino acids, and a maximum of two miscleavages were allowed. Peptide and protein level false-discovery rates (FDRs) were filtered to 1% using the target-decoy strategy. Protein tables were filtered to eliminate the identifications from the reverse database and common contaminants. Identification parameters also included a minimum of two unique peptides for identification. The data were quantified by label-free analysis using the same software, based on extracted ion currents (XICs) of peptides, enabling quantitation from each LC-MS run for each peptide identified in any of the experiments. Briefly, for every peptide, corresponding total signals from multiple runs were compared to determine peptide ratios. Pairwise peptide ratios were determined only when the corresponding peak was detected in both LC-MS runs. A robust estimation of the protein ratio was calculated as the median of pairwise peptide ratios interpolated with the square root of the ratio of summed intensities. The analysis was done after first recalibrating the retention times. Data transformation ($\log_2$) and statistical tests were done using Preseus 1.4.0.20 software.

RT-PCR. RNA was extracted from *Salmonella* cultures grown to late logarithmic phase at 37°C using the Qiagen RNAprotect Bacteria Reagent and the RNeasy minikit (Qiagen) according to the manufacturer's instructions, including an on-column DNase digestion. The purified RNA was secondarily treated with RNase-free DNase I, followed by ethanol precipitation, and 200 ng of DNase I-treated RNA was subjected to first-strand cDNA synthesis, using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the kit protocol. Real-time (RT) PCRs were performed in an Applied Biosystems 7500 Fast real-time PCR system. Each reaction was carried out in a total volume of 20 μl on a 96-well optical reaction plate (Applied Biosystems) containing 10 μl FastStart Universal SYBR green Master (ROX) mix (Roche Applied Science), 2 μl cDNA, and two gene-specific primers at a final concentration of 0.3 μM each (see Table S2 in the supplemental material). The real-time cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting-curve analysis verified that each reaction mixture contained a single PCR product. Relative quantification of transcripts was evaluated using the comparative threshold cycle (C_T) method. The housekeeping gene *rpoD* was used as the endogenous normalization control. The ΔC_T values were calculated by determining the difference in threshold values for the target gene and *rpoD* in *S.* Typhimurium SL1344 versus *S.* Paratyphi A 45157 or the wild type versus the mutant strain. Calculation of $\Delta\Delta C_T$ involved the subtraction of the normalized wild-type ΔC_T value from the normalized ΔC_T value of the compared mutant. Fold differences in gene expression were calculated as $2^{-\Delta\Delta CT}$.

RESULTS

Typhoidal serovars are less motile than *S.* Typhimurium. Considering that at least four inactivated genes are present in the motility-chemotaxis regulon in *S.* Paratyphi A (10), we were interested in comparing the motility phenotype of *S.* Paratyphi A to that of *S.* Typhimurium. We analyzed the swimming motilities on soft (0.3%) agar plates of 3 reference (sequenced) strains and 12 randomly selected clinical (human) isolates of *S.* Typhimurium and *S.* Paratyphi A (15 strains from each serovar). Although some degree of intraserovar variation was seen, *S.* Typhimurium strains presented significantly higher motility, with a mean swimming radius of 28 ± 5 mm (Fig. 1), than *S.* Paratyphi A, with a mean swimming radius of only 10 ± 3 mm. To extend this characterization to other typhoidal serovars, further clinical strains of *S.* Sendai ($n = 4$) and *S.* Typhi ($n = 7$) were analyzed in comparison to the poultry-specific typhoidal serovars *Salmonella enterica* serovar

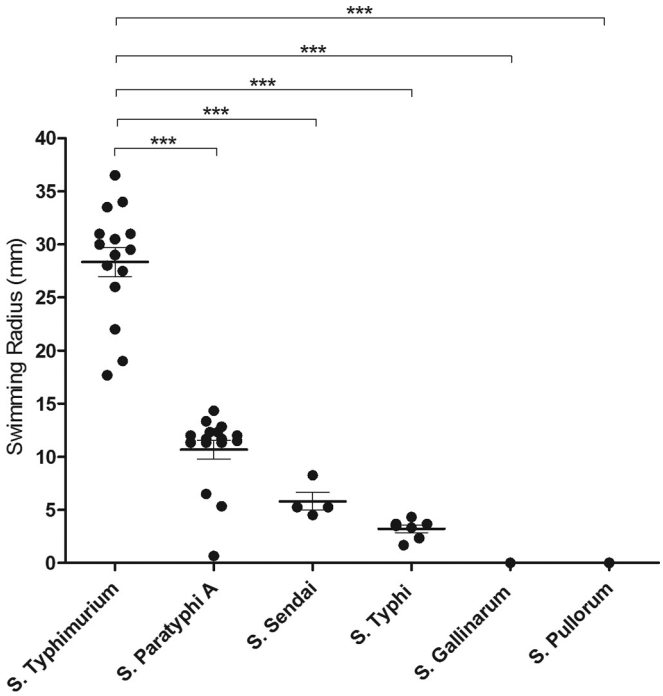

FIG 1 Typhoidal serovars are less motile than *S.* Typhimurium. Ten microliters of overnight cultures from 15 strains of *S.* Typhimurium (SL1344, LT2, 14028S, 92576, 80609, 74701, 73727, 88359, 78651, 88894, 92273, 116449, 103259, 9866, and 82788), 15 strains of *S.* Paratyphi A, (45157, 9150, AKU12061, 83698, 83753, 93223, 108003, 108599, 124597, 119989, 113498, 105493, 51190, 36056/7, and 45842/7), 4 strains of *S.* Sendai (55-2461, SKT 71, 830431, and 741035), 7 strains of *S.* Typhi (CT18, 120130191, 152496, 153029, 152599, 152201, and 152599), *S.* Gallinarum (287/91), and *S.* Pullorum were inoculated onto soft (0.3%) agar plates and incubated at 37°C for 5.5 h. Each dot represents the mean swim halo radius of one strain (with 2 or 3 biological repeats). The mean swimming radius and the standard error of the mean (SEM) for each serovar are shown by a horizontal line and error bars, respectively. ***, $P < 0.0001$.

Pullorum and *Salmonella enterica* serovar Gallinarum, known to be nonmotile (17). The examined strains of *S.* Sendai and *S.* Typhi were even less motile than *S.* Paratyphi A and presented mean swimming radii of only 6 ± 1 mm and 3 ± 1 mm, respectively. This analysis indicated that all three of the typhoidal human-restricted salmonellae present a reduced motility phenotype compared to the nontyphoidal *Salmonella* (NTS) serovar *S.* Typhimurium.

***S.* Paratyphi A expresses a lower level of the flagellar regulon than *S.* Typhimurium.** A possible explanation of the restrained motility of *S.* Paratyphi A relative to *S.* Typhimurium that was considered was a lower level of expression of the motility-chemotaxis genes in the former. In *Salmonella*, the flagellar, motility, and chemotaxis genes constitute a hierarchical regulon of more than 60 genes organized into three classes that are temporally expressed in a cascade. Therefore, the expression of one class of genes is required for the transcription of the subsequent class of genes (18). At the top of the hierarchy is a class I master transcriptional complex, $FlhD_4C_2$, encoded by the single operon *flhDC*, necessary for the activation of *fliA* and other class II genes. FliA, in turn, is required for the expression of the third class of genes (see Fig. S1 in the supplemental material).

Transcriptome analysis (RNA-Seq) of *S.* Typhimurium (strain

TABLE 1 Transcription of the motility-chemotaxis regulon in *S.* Paratyphi A and *S.* Typhimurium[a]

Locus Tag	Gene Name	Function	STM	SPA	Log_2 FC	FDR
STM1171	*flgN*	Flagellar biosynthesis protein FlgN	9.47	7.47	-2.00	0.000
STM1172	*flgM*	Negative regulator of flagellin synthesis FlgM	9.66	7.67	-1.99	0.001
STM1173	*flgA*	Flagellar basal-body P-ring formation protein FlgA	8.48	5.53	-2.94	0.000
STM1174	*flgB*	Flagellar basal-body rod protein FlgB	9.18	7.05	-2.13	0.001
STM1175	*flgC*	Flagellar basal-body rod protein FlgC	9.50	7.09	-2.41	0.002
STM1176	*flgD*	Flagellar basal-body rod modification protein FlgD	9.86	7.46	-2.40	0.001
STM1177	*flgE*	Flagellar hook protein FlgE	10.78	8.27	-2.51	0.000
STM1178	*flgF*	Flagellar basal-body rod protein FlgF	9.96	7.17	-2.79	0.000
STM1179	*flgG*	Flagellar basal-body rod protein FlgG	9.73	7.01	-2.73	0.000
STM1180	*flgH*	Flagellar L-ring protein FlgH	8.68	6.06	-2.62	0.000
STM1181	*flgI*	Flagellar P-ring protein FlgI	8.72	6.03	-2.69	0.000
STM1182	*flgJ*	Flagellar protein FlgJ [peptidoglycan hydrolase] (EC 3.2.1.-)	8.92	6.18	-2.74	0.000
STM1183	*flgK*	Flagellar hook-associated protein FlgK	10.82	8.45	-2.37	0.000
STM1184	*flgL*	Flagellar hook-associated protein FlgL	10.60	8.32	-2.28	0.000
STM1626	***trg***	**Methyl-accepting chemotaxis protein III (ribose and galactose chemoreceptor protein)**	9.25	-3.01	-12.27	0.000
STM1912	*flhE*	Flagellar protein FlhE	7.28	4.89	-2.39	0.000
STM1913	*flhA*	Flagellar biosynthesis protein FlhA	8.49	5.64	-2.85	0.000
STM1914	*flhB*	Flagellar biosynthesis protein FlhB	7.21	4.56	-2.65	0.000
STM1915	*cheZ*	Chemotaxis response - phosphatase CheZ	9.50	8.47	-1.03	0.020
STM1916	*cheY*	Chemotaxis regulator - transmits chemoreceptor signals to flagelllar motor components CheY	9.33	8.24	-1.09	0.018
STM1917	*cheB*	Chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61)	8.34	7.08	-1.26	0.011
STM1918	*cheR*	Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)	8.55	6.93	-1.62	0.001
STM1919	***cheM/tar***	**Methyl-accepting chemotaxis protein**	10.22	8.09	-2.13	0.000
STM1920	*cheW*	Positive regulator of CheA protein	9.67	8.28	-1.38	0.006

(Continued on following page)

SL1344) and *S.* Paratyphi A (strain 45157) revealed ~5-fold-lower expression over the entire flagellar regulon in *S.* Paratyphi A than in *S.* Typhimurium (Table 1). Quantitative RT (qRT)-PCR analyses of *flhD* (class I), *fliZ* and *flgM* (class II), and *cheA* and *fliC* (class III) confirmed the transcriptome analysis and showed that motility genes were expressed in *S.* Paratyphi A at 6- to 55-fold-lower levels than in *S.* Typhimurium (Fig. 2A). In addition, we determined the protein levels of FliC in three *S.* Typhimurium and three *S.* Paratyphi A representative strains. Western blotting against FliC tagged with a 2HA epitope demonstrated significantly lower levels of flagellin in all of these *S.* Paratyphi A strains than in *S.* Typhimurium strains (Fig. 2B). These results were correlated with the impaired motility of *S.* Paratyphi A and indicated that the entire motility-chemotaxis regulon is expressed at lower levels in *S.* Paratyphi A than in *S.* Typhimurium.

Flagella are required for *S.* Paratyphi A entry into host cells. Previous studies from several groups have suggested that the flagella are involved in virulence-associated phenotypes of particular *S. enterica* serovars (19–22), but not in the virulence of *S.* Gallinarum, which naturally lacks flagella (23). Considering the genome degradation in the flagellar regulon and its lower expression in *S.* Paratyphi A, we next asked if flagella are actually required for host cell adhesion and invasion by *S.* Paratyphi A in comparison to *S.* Typhimurium. Nonpolar deletions of *flhA* (a structural gene of the flagellum basal body) and *fliC* (phase I flagellin) were constructed in *S.* Paratyphi A (strain 45157) and in *S.* Typhimurium (strain SL1344). To evaluate the effects of these deletions on motility, the *fliC* and *flhA* mutants were tested in a swimming assay on soft agar plates. As expected, while the deletion of *flhA* completely abolished motility in both serovars (as flagella could not be assembled), the deletion of *fliC* reduced the motility of *S.* Typhimurium to about 50% but resulted in a completely nonmotile phenotype in *S.* Paratyphi A (Fig. 3A). The different effects of a *fliC* mutation in the two serovars is most likely due to the monophasic nature of *S.* Paratyphi A and its inability to express the alternative flagellin subunit FljB in the absence of FliC.

To assess the role of the flagella in host cell adhesion, infection assays in the presence of CD (a drug that inhibits actin polymerization and subsequently *Salmonella* invasion) were implemented. These experiments showed that the *fliC* and *flhA* mutant

TABLE 1 (Continued)

Locus Tag	Gene Name	Function	STM	SPA	Log_2 FC	FDR
		activity (CheW)				
STM1921	*cheA*	Signal transduction histidine kinase CheA (EC 2.7.3.-)	10.64	9.42	-1.22	0.016
STM1922	*motB*	Flagellar motor rotation protein MotB	9.61	7.92	-1.69	0.001
STM1923	*motA*	Flagellar motor rotation protein MotA	9.26	7.67	-1.59	0.002
STM1924.S	*flhC*	Flagellar transcriptional activator FlhC	8.76	7.47	-1.29	0.000
STM1925	*flhD*	Flagellar transcriptional activator FlhD	9.04	7.86	-1.19	0.000
STM1954	*fliY*	Cystine ABC transporter, periplasmic cystine-binding protein FliY	8.84	8.30	-0.54	0.127
STM1955	*fliZ*	Flagellar biosynthesis protein FliZ	9.35	7.44	-1.91	0.001
STM1956	*fliA*	RNA polymerase sigma factor for flagellar operon	9.80	7.86	-1.94	0.002
STM1958	***fliB***	**Lysine-N-methylase (EC 2.1.1.-)**	8.85	5.95	-2.90	0.000
STM1959	*fliC*	Flagellar biosynthesis protein FliC	13.19	12.05	-1.14	0.052
STM1960	*fliD*	Flagellar hook-associated protein FliD	10.79	9.25	-1.54	0.005
STM1961	*fliS*	Flagellar biosynthesis protein FliS	9.75	8.25	-1.50	0.004
STM1962	*fliT*	Flagellar biosynthesis protein FliT	9.50	8.12	-1.38	0.003
STM1968	*fliE*	Flagellar hook-basal body complex protein FliE	6.73	3.29	-3.44	0.000
STM1969	*fliF*	Flagellar M-ring protein FliF	9.74	6.29	-3.45	0.000
STM1970	*fliG*	Flagellar motor switch protein FliG	9.69	6.59	-3.11	0.000
STM1971	*fliH*	Flagellar assembly protein FliH	9.20	6.23	-2.97	0.000
STM1972	*fliI*	Flagellum-specific ATP synthase FliI	8.82	5.60	-3.21	0.000
STM1973	*fliJ*	Flagellar protein FliJ	8.73	5.83	-2.89	0.000
STM1974	*fliK*	Flagellar hook-length control protein FliK	9.63	6.74	-2.89	0.000
STM1975	*fliL*	Flagellar biosynthesis protein FliL	9.00	6.60	-2.40	0.000
STM1976	*fliM*	Flagellar motor switch protein FliM	9.98	7.57	-2.40	0.000
STM1977	*fliN*	Flagellar motor switch protein FliN	9.40	6.83	-2.57	0.000
STM1978	*fliO*	Flagellar biosynthesis protein FliQ	8.60	6.01	-2.60	0.000
STM1979	*fliP*	Flagellar biosynthesis protein FliP	8.28	6.12	-2.15	0.000
STM1980	*fliQ*	Flagellar biosynthesis protein FliQ	7.13	5.35	-1.79	0.000
STM1981	*fliR*	Flagellar biosynthesis protein FliR	6.46	4.42	-2.04	0.000
STM2314	*cheV*	Chemotaxis protein CheV (EC 2.7.3.-)	10.18	8.15	-2.03	0.000

[a] Columns STM (*S.* Typhimurium) and SPA (*S.* Paratyphi A) represent averaged FPKM (fragments per kilobase per million reads) values on a log_2 scale for motility genes assayed from two independent replicates expressed during the late log phase in *S.* Typhimurium SL1344 and *S.* Paratyphi A 45157, respectively. Columns Log_2FC and FDR represent the log_2 fold change of *S.* Paratyphi A compared to *S.* Typhimurium and the associated false-discovery rate, respectively. The genes in boldface represent features that are pseudogenes in *S.* Paratyphi A.

strains of *S.* Typhimurium (Fig. 3B) and *S.* Paratyphi A (Fig. 3C) adhered to human epithelial CaCo-2 cells at levels similar to those of their parental wild-type strains, suggesting that the flagella do not play a role in epithelial cell adhesion.

Nonetheless, the capabilities of the *S.* Typhimurium (Fig. 3D) and *S.* Paratyphi A (Fig. 3E) *fliC* and *flhA* strains to invade CaCo-2 cells were severely reduced, by more than 20-fold, compared to the wild type. Complementation of *flhA* and *fliC* from a low-copy-number plasmid (pWSK29) restored most (70 to 100%) of the invasive abilities of the mutant strains in both serovars. Similar results were obtained with HeLa cells (data not shown; see Fig. S3 in the supplemental material), indicating that this phenotype is not cell line specific.

We next investigated the role of flagella in phagocytic cell uptake. As with the epithelial cells, although the absence of *flhA* or *fliC* did not lessen *Salmonella* adherence to J774 macrophage-like cells (data not shown), *flhA* and *fliC* mutants of *S.* Typhimurium (Fig. 4A) or *S.* Paratyphi A (Fig. 4B) entered macrophages at significantly lower levels than their parental strains. Complementation of *flhA* and *fliC* restored macrophage uptake to levels similar to those of the wild type. Reduced macrophage uptake of these *S.* Typhimurium and *S.* Paratyphi A mutants was also found in differentiated human macrophages and THP-1 cells (Fig. 4C and D, respectively). We concluded from these experiments that flagella are required for optimal macrophage entry by both *S.* Paratyphi A and *S.* Typhimurium.

To exclude the possibility that the impaired macrophage uptake is due to the role of flagellin as a pathogen-associated molecular pattern (PAMP) recognized by pattern recognition receptors, such as Toll-like receptor 5 (TLR-5) (24), and that uptake does not require functional flagella, we repeated these experiments in the presence of purified *S.* Typhimurium flagellin. As illustrated in Fig. 4E, adding exogenous flagellin, in concentrations (1 μg/ml) that were shown to activate TLR-5 (25), to THP-1 (Fig. 4E) or J774 (data not shown) macrophages did not induce *Salmonella* uptake in the absence of intact FliC, showing that functional flagella are required for *Salmonella* entry into phagocytic cells.

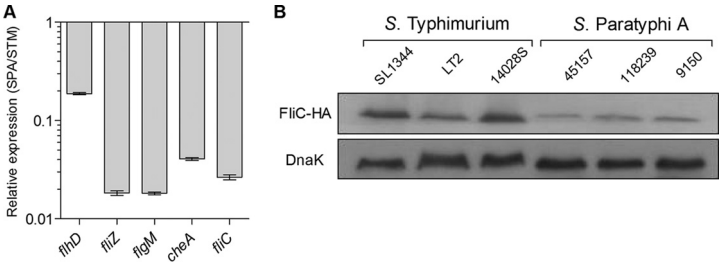

FIG 2 *S.* Paratyphi A expresses lower levels of flagellar genes than *S.* Typhimurium. (A) Total RNA was harvested from *S.* Typhimurium SL1344 (STM) and *S.* Paratyphi A45157 (SPA) cultures grown to late logarithmic phase at 37°C and subjected to qRT-PCR. The fold changes in the abundances of the *flhD*, *fliZ*, *flgM*, *cheA*, and *fliC* transcripts (normalized to *rpoD*) in *S.* Paratyphi A relative to their expression in *S.* Typhimurium are shown. The indicated values present the means and SEM of the results of three independent RT-PCR experiments from two independent RNA extractions. (B) SDS-PAGE Western blot analysis of bacterial cell lysates from three *S.* Typhimurium and three *S.* Paratyphi A strains expressing FliC-2HA grown to the late logarithmic phase. Protein fractions were probed using anti-HA antibody with anti-DnaK as a control.

The absence of flagellin induces SPI-1 expression in *S.* Paratyphi A. The results presented above suggested a functional link between flagella and host cell entry by *Salmonella*. One of the main mechanisms required for active eukaryotic cell invasion by *Salmonella* is the T3SS encoded within SPI-1 and its designated secreted protein effectors (6). In order to better understand a possible effect of flagellar absence on SPI-1, we quantified the expression of SPI-1 structural (*invA*), regulatory (*invF*), and translocated effector (*sipB*, *sopB*, and *sopE2*) genes in the absence of *fliC* compared to the wild-type strain in *S.* Typhimurium and *S.* Paratyphi A. Surprisingly, this analysis demonstrated a 4- to 8-fold increase in the transcripts of the above-mentioned SPI-1 genes in the absence of *fliC* in *S.* Paratyphi A, but not in *S.* Typhimurium, that even presented subtle repression (Fig. 5A). Western blotting against SopB (effector protein) and PrgJ (structural protein) tagged with a hemagglutinin epitope confirmed the transcription results and demonstrated an elevated level of these T3SS-1 proteins in the cellular fraction of *S.* Paratyphi A in the absence of FliC, but not in *S.* Typhimurium (Fig. 5B).

To study the effect of the lack of FliC on the secretion of T3SS-1 effectors, biochemical and proteomics approaches were taken. *Salmonella* cultures were grown under SPI-1 induction conditions (26) to the late logarithmic phase in LB medium, and the filtered cell-free supernatant was precipitated by TCA, followed by SDS-PAGE. In close agreement with the qRT-PCR and the Western blot analyses, we showed that in the absence of FliC, the secretion of multiple proteins with the expected molecular weights of SipA, SigD, SipC, InvJ, and SopE were significantly induced compared to the wild-type background. In addition to the oversecretion of SPI-1 effectors, we also observed that the lack of FliC resulted in excess export of flagellar components (including FlgK, FliD, and FlgL), while *fliC* complementation or expression of *S.* Typhimurium *fljB* in *S.* Paratyphi A counteracted this phenomenon (Fig. 5C). Furthermore, oversecretion of SPI-1 and flagellar proteins was not observed in an *flhA* background (Fig. 5D), indicating that the lack of flagellin specifically and not the absence of the flagellar apparatus results in overexpression and secretion of SPI-1 and flagellar proteins. To further confirm that this oversecretion of SPI-1effectors is indeed a controlled export via a T3SS and not a spillover of proteins from the cell, we also performed this assay using an *S.* Paratyphi A *fliC invA ssaR* triple-mutant strain. Oversecretion of SPI-1 effectors was abolished in the *fliC invA ssaR* mutant (see Fig. S2 in the supplemental material), indicating that it is indeed a T3SS-dependent process, rather than uncontrolled protein leakage.

To further characterize this phenomenon, we applied a quantitative proteomics approach and analyzed by LC–MS-MS the secretomes of *S.* Paratyphi A and *S.* Typhimurium relative to their isogenic *fliC* mutants under SPI-1-inducing conditions. As expected, the most abundant secreted proteins were the *Salmonella* SPI-1 effectors, including SopE2, SopE, SipB, SipA, SopB, SteA, SipD, SopD, and SipC. Two independent quantitative-proteomics experiments clearly showed that the levels of all of the identified SPI-1 effectors secreted into the medium were 2.2- to 17-fold higher in the *fliC* background than in the wild type in *S.* Paratyphi A, but not in *S.* Typhimurium. Similar results were also obtained for many of the secreted flagellar components (Table 2). Other peptidoglycan-associated lipoproteins, such as DnaK and GroEL (27), were identified in similar amounts, suggesting that the increased secretion is specific to the exported flagellar and SPI-1 proteins.

Flagellin affects the expression of *S.* Paratyphi A motility and SPI-1 regulons in a FliZ-dependent manner. To better understand how the absence of flagellin affects the expression of SPI-1 and the motility regulons, we determined the transcriptional levels of four motility (*flhD*, *fliA*, *fliZ*, and *flgM*) and two SPI-1 (*hilA* and *hilD*) regulators. This analysis showed a significant induction of these six regulatory genes in the absence of FliC only in *S.* Paratyphi A and not in *S.* Typhimurium (Fig. 6A) and indicated that the transcriptional upregulation of these regulons starts at least at the level of the master regulators FlhDC and HilD. Ectopic complementation of either *fliC* (*S.* Paratyphi A) or *fljB* (*S.* Typhimurium) in the *S.* Paratyphi A *fliC* background counteracted the induction of the motility genes to levels similar to those of the wild type (or even below when *fljB* was expressed) (Fig. 6B). Similarly, the induced transcription of SPI-1 genes (*invF*, *invA*, *sipB*, *sopB*, and *sopE2*) in the *S.* Paratyphi A *fliC* mutant was also greatly decreased following expression of *fliC* or *fljB* (Fig. 6C). These results indicated that both types of flagellin (FliC and FljB) can affect the expression of the flagellar and SPI-1 regulons at the transcriptional level in *S.* Paratyphi A.

Previous studies have shown in *S.* Typhimurium a regulatory linkage between the flagella and SPI-1 via FliZ, which controls the positive regulators of SPI-1, HilD, and HilA posttranscriptionally (28–30). To investigate the possibility that in *S.* Paratyphi A FliC affects SPI-1 gene expression via FliZ, we

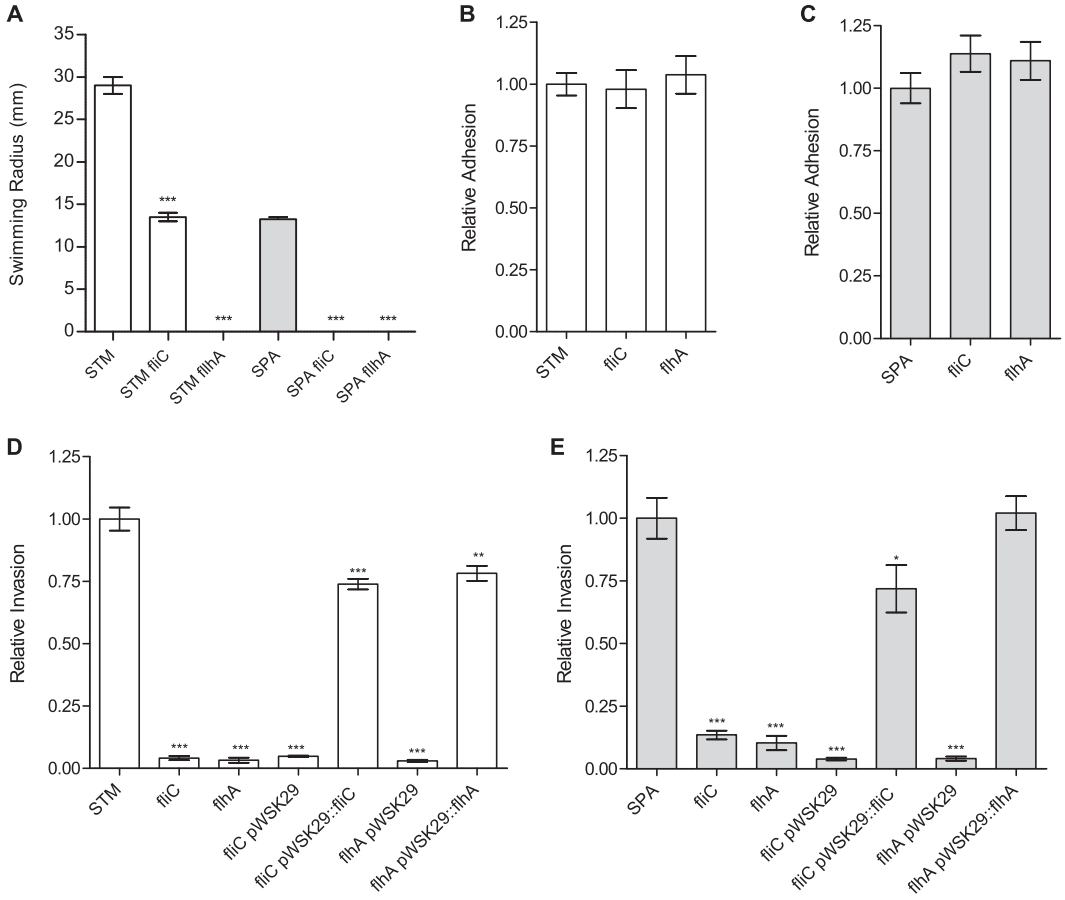

FIG 3 Flagella are required for *S.* Paratyphi A and *S.* Typhimurium invasion into epithelial cells. (A) Ten microliters of overnight cultures of *S.* Typhimurium strain SL1344 (STM), *S.* Paratyphi A strain 45157 (SPA), and their isogenic *fliC*- and *flhA*-null mutant strains were inoculated onto soft (0.3%) agar plates and incubated at 37°C for 5.5 h. (B and C) *Salmonella* strains were grown in LB medium under microaerophilic conditions and used to infect Caco-2 cells. *S.* Typhimurium (B) and *S.* Paratyphi A (C) adhesion was determined in the presence of cytochalasin D and is shown as the percentage of cell-associated bacteria from the total number of bacteria used to infect the cells. (D and E) *S.* Typhimurium (D) and *S.* Paratyphi A (E) invasion was determined at 2 h p.i using a gentamicin protection assay and calculated as the percentage of intracellular bacteria (CFU) recovered at 2 h p.i from the total number of CFU used to infect the cells. Under these conditions, the invasion proportions in the wild-type backgrounds were 2.3% and 2.6% of the infecting inocula of *S.* Typhimurium and *S.* Paratyphi A, respectively. The mutant strains were complemented with vector (pWSK29) only or with *fliC* or *flhA* cloned into pWSK29. The data represent the means and SEM of the results of at least three biological replicates. *Salmonella* mutant strains are shown relative to their corresponding wild-type backgrounds. Analysis of variance (ANOVA) with Dunnett's multiple-comparison test was used to determine differences between data sets. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

constructed a *fliC fliZ S.* Paratyphi A double-mutant strain and determined the transcriptional levels of representative genes from the flagella, including *flhD* (class I), *fliA* and *flgM* (class II), and *cheA* (class III), and from SPI-1, *hilD*, *hilA*, and *invF* (regulators), *invA* (T3SS-1 structural gene), and *sopB* (secreted effector), in the absence of both FliC and FliZ. Quantitative RT-PCR analysis clearly showed that in the *fliC fliZ* background these SPI-1 and flagellar genes are not induced (Fig. 7A). In close agreement with the transcriptional analysis, we were further able to show that the oversecretion of SPI-1 effectors that was found in the absence of FliC in *S.* Paratyphi A (Fig. 5) was significantly repressed in a *fliC fliZ* mutant strain of *S.* Paratyphi A (Fig. 7B). We concluded from these analyses that in *S.* Paratyphi A, SPI-1 and flagellar regulon induction in the absence of flagellin is mediated by the class II regulator FliZ.

To further study the effect of such a regulatory change on *S.* Paratyphi A invasion, we compared the invasion capabilities of *fliC*, *fliZ*, and *fliC fliZ* mutants to the invasion of T3SS-1 mutant strains harboring a null deletion in *invA* or *invG*. Although the absence of FliC led to upregulation of SPI-1 expression in *S.* Paratyphi A, a *flic* mutant showed epithelial cell invasion that was similar to the poor invasion of *invA* or *invG* strains and lower than the invasion in a *fliZ* background (see Fig. S3 in the supplemental material).

Since *S.* Typhimurium expresses two flagellin subunits (FliC and FljB), we next asked if SPI-1 and flagellar gene induction also occurs in *S.* Typhimurium lacking both types of flagellin. Interestingly, *S.* Typhimurium lacking both *fliC* and *fljB* did not show oversecretion of SPI-1 and flagellar proteins (Fig. 7C) and did not present any transcriptional induction of SPI-1 or flagellar genes. Instead, a moderate reduction in the transcription of these genes was observed in the *S.* Typhimurium *fliC fljB* background (Fig. 7D). These data clearly demonstrated that the effect of flagellin absence resulting in upregulation of SPI-1 and the motility genes is specific to *S.* Paratyphi A and does not occur in *S.* Typhimurium. Altogether, these results demonstrated, for the first time, that SPI-1 and flagellar gene expression (and, consequently, effector secretion) is affected by flagellin in *Salmonella*.

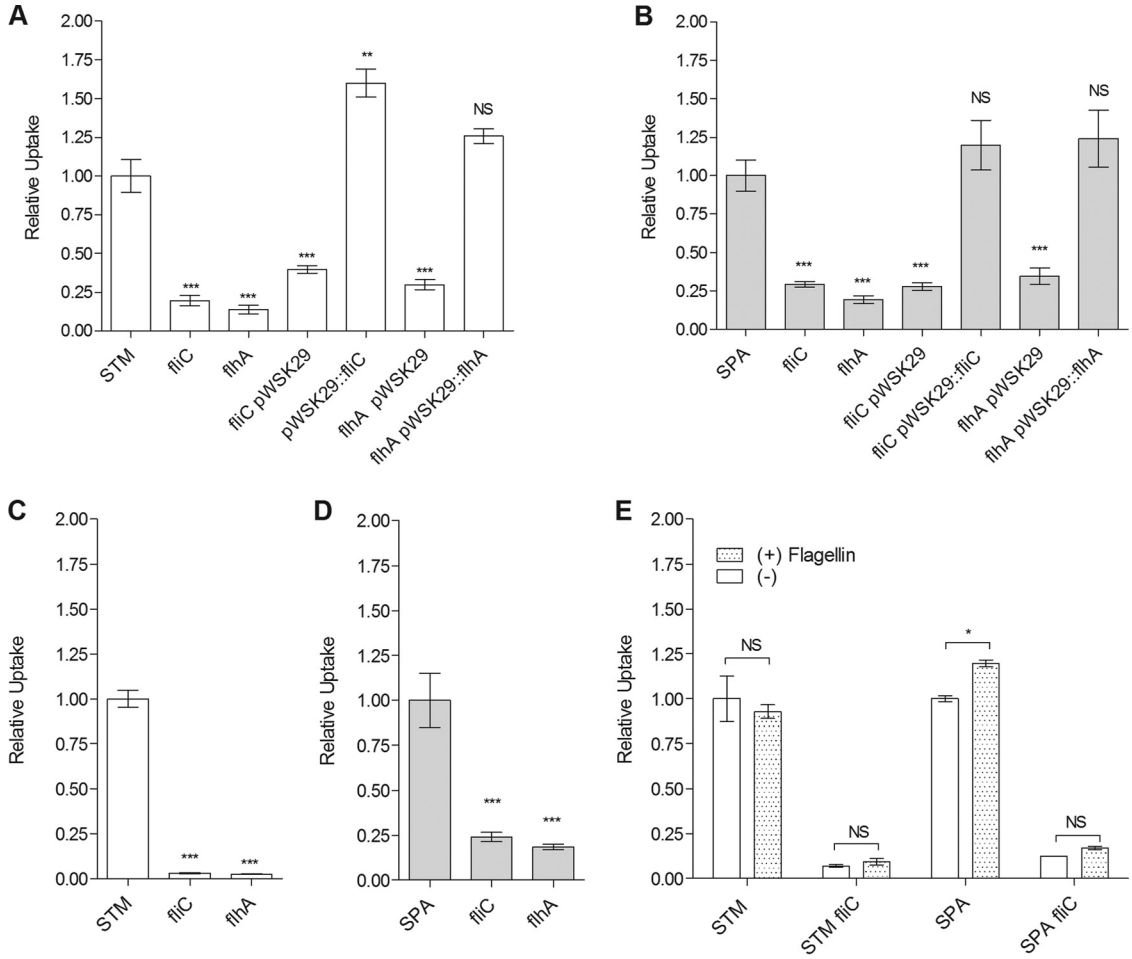

FIG 4 Flagella are required for *S.* Paratyphi A and *S.* Typhimurium macrophage uptake. (A to D) *S.* Typhimurium strain SL1344 (STM) (A), *S.* Paratyphi A strain 45157 (SPA) (B), their isogenic *fliC*- and *flhA*-null mutant strains, and mutant strains complemented with vector (pWSK29) or with the gene *fliC* or *flhA* cloned into pWSK29 were compared for their abilities to enter J744.1A mouse-derived macrophages (A and B) and THP-1 human-derived macrophages (C and D) using the gentamicin protection assay. Uptake is shown relative to the wild-type strain. Under these conditions, THP-1 phagocytosis of the wild type was 2.6% and 2.4% of the infecting inocula of *S.* Typhimurium and *S.* Paratyphi A, respectively. (E) THP-1 cells were pretreated with high-purity flagellin (0.1 μg/ml), followed by the gentamicin protection assay as for panels A to D. *Salmonella* uptake is shown relative to untreated cells. The results represent the means of the results of three independent experiments, with SEM shown by the error bars. ANOVA with Dunnett's multiple-comparison test was used to determine differences between data sets. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; NS, not significant.

DISCUSSION

Salmonella flagella have long been known to play multiple key roles in bacterial physiology and virulence (9). Considering the different interactions of NTS and *S.* Paratyphi A with the human host (1) and the degradation of multiple motility-chemotaxis genes in the *S.* Paratyphi A genome (10), we sought to characterize the motility phenotype and the role of flagella in *S.* Paratyphi A virulence-associated phenotypes in comparison to the ubiquitous NTS serovar *S.* Typhimurium.

Although *S. enterica* serovars are generally considered to be motile bacilli (31), we found substantial differences in the motilities of serovars important to human health. Multiple sequenced model strains and clinical low-passage-number isolates of *S.* Paratyphi A, *S.* Typhi, and *S.* Sendai were found to be considerably less motile than *S.* Typhimurium (Fig. 1). Previously, the *S.* Typhi-specific regulator TviA was shown to repress genes involved in flagellum-mediated motility (32, 33). However, our results show that all three of the typhoidal serovars, including those that do not harbor the *tviA* locus, are less motile than *S.* Typhimurium. This observation suggests a distinct phenotype common to the typhoidal host-restricted serovars that are motility impaired (*S.* Paratyphi A, *S.* Typhi, and *S.* Sendai) or completely nonmotile, like the avian-restricted serovars *S.* Gallinarum and *S.* Pullorum. In agreement with these results, RT-PCR, Western blotting, and comparative RNA-Seq have demonstrated lower expression of the motility-chemotaxis regulon in *S.* Paratyphi A strains than in *S.* Typhimurium strains (Fig. 2 and Table 1), which may explain their reduced motility.

The role of flagella in *Salmonella* virulence has been studied in several *S. enterica* serovars, including *S.* Typhi (21), *S.* Typhimurium (19, 34, 35), *Salmonella enterica* serovar Enteritidis (20, 36, 37), *Salmonella enterica* serovar Dublin (22), and *S.* Gallinarum (23), but thus far, not in *S.* Paratyphi A. Having established differences in the motility phenotypes (Fig. 1) and in the expression of the entire flagellar regulon between *S.* Typhimurium and *S.* Paratyphi A (Fig. 2 and Table 1), we were interested in comparing the

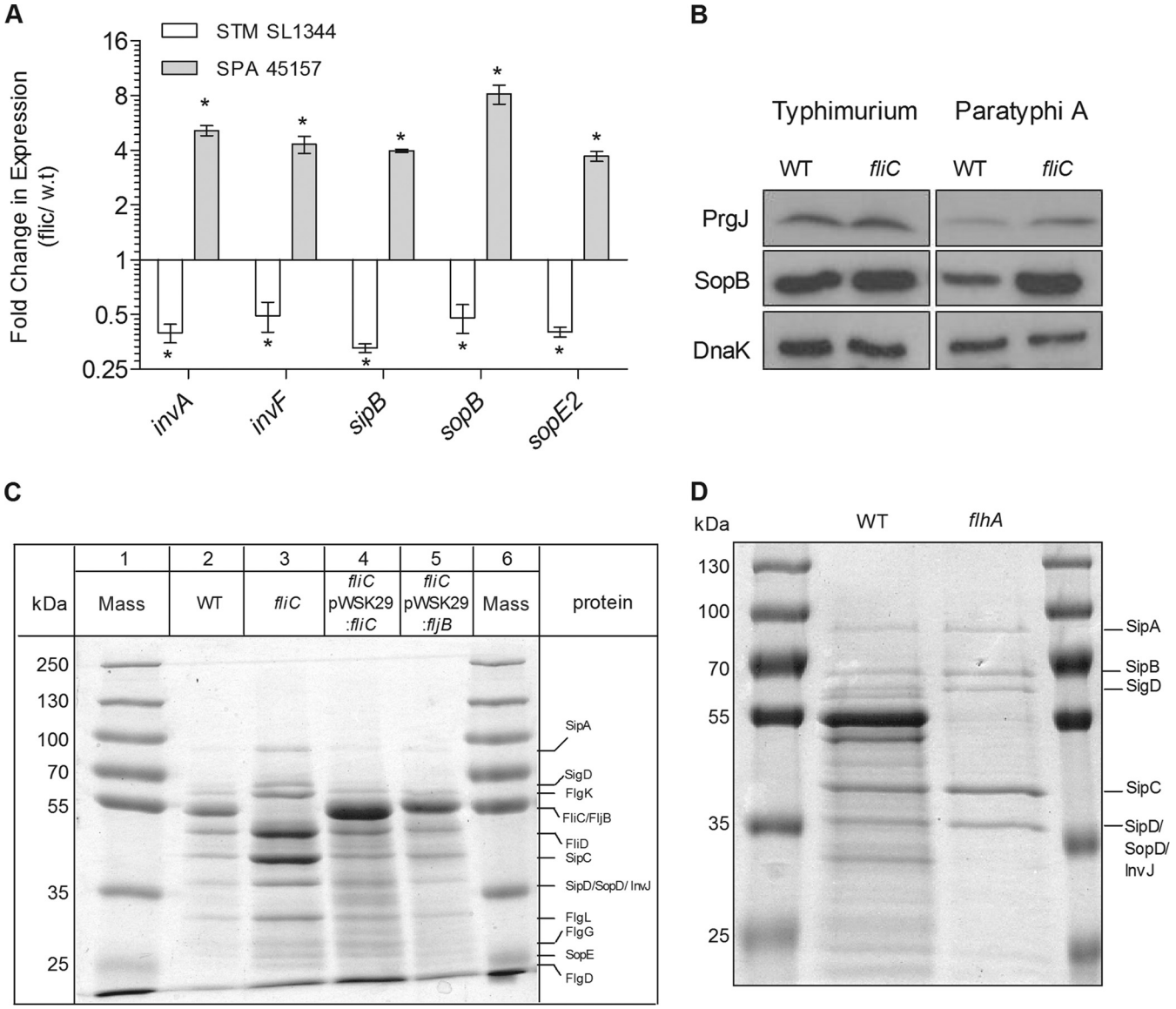

FIG 5 The absence of flagellin induces SPI-1 gene expression in *S.* Paratyphi A. (A) Total RNA was harvested from *S.* Typhimurium SL1344 and *S.* Paratyphi A 45157 wild-type and *fliC* mutant cultures grown to late logarithmic phase in LB medium and was subjected to qRT-PCR. The fold changes in the abundances of the *invA*, *invF*, *sipB*, *sopB*, and *sopE2* transcripts (normalized to *rpoD*) in the *fliC* background in *S.* Typhimurium or *S.* Paratyphi A relative to their expression in the wild type are shown. The indicated values represent the means and SEM of the results of three independent RT-PCR experiments from two independent RNA extractions. A two-tailed one-sample *t* test was used to determine statistical significance (*). (B) SDS-PAGE Western blot analysis of bacterial cell lysates from *S.* Typhimurium and *S.* Paratyphi A wild type (WT) and a *fliC* mutant expressing SopB-2HA or PrgJ-2HA. Protein fractions were probed using anti-HA antibodies with anti-DnaK as a loading control. (C) *S.* Paratyphi A cultures that were grown in LB medium for 5.5 h were normalized to an OD_{600} of 2.4 to 2.6, and their supernatant proteins were precipitated by TCA. Equal amounts (25 μl) of the precipitated fractions from *S.* Paratyphi A wild type (lane 2), *fliC* (lane 3), *fliC* complemented with pWSK29::$fliC_{SPA}$ (lane 4), and *fliC* complemented with pWSK29::$fljB_{STM}$ (lane 5) were separated on SDS-12% polyacrylamide gels and stained with Coomassie G-250. (D) Precipitated protein fractions (25 μl) from equal volumes of OD-normalized *S.* Paratyphi A wild-type and *flhA* cultures were separated on SDS-polyacrylamide gels and stained as for panel C.

roles of flagella in their virulence-associated phenotypes. *S.* Typhimurium and *S.* Paratyphi A lacking *fliC* or *flhA* showed the same degree of adhesion to macrophages (data not shown) and Caco-2 cells as the wild-type parental strains (Fig. 3B and C), indicating that flagella do not play a role in adhesion to these cells. These results are in conflict with the reported roles for *S.* Dublin and *S.* Typhimurium flagella in bacterial adherence (22) but agree with the dispensable role of FliC in host cell adhesion of *S.* Enteritidis (37). In contrast to adhesion, we demonstrated that the absence of both FliC and FlhA dramatically reduced the abilities of both of *S.* Typhimurium and *S.* Paratyphi A to invade epithelial cells (Fig. 3D and E) and to enter macrophages (Fig. 4). These results are consistent with previous studies showing that flagella from other serovars, including *S.* Typhimurium (22), *S.* Enteritidis (37), and *S.* Dublin (22), are required for active invasion of epithelial cells and macrophage uptake, suggesting a universal role of flagella in host cell invasion in all of these serovars. Complementing the expression of FliC or FlhA, but not supplementing exogenous

TABLE 2 Effects of FliC absence on the secretomes of *S.* Paratyphi A and *S.* Typhimurium[a]

Protein Name	Function	SPA tag	STM tag	SPA fliC to WT ratio		STM fliC to WT ratio	
				exp. 1	exp. 2	exp. 1	exp. 2
InvJ	T3SS-1 needle regulator	SPA2750	STM2892	10	6.1	2.2	1.7
SopD	SPI-1 effector	SPA2801	STM2945	5.9	5.6	1.7	1
SopE	SPI-1 effector	SPA2564	STM1855	9.5	5.3	0.8	1
PrgI	T3SS-1 needle	SPA2731	STM2873	4.2	5.2	0.6	1.1
PrgJ	T3SS-1 rod	SPA2730	STM2872	2.2	4.9	0.6	1
SipC	SPI-1 effector	SPA2742	STM2884	8	4.7	2	2
SipA	SPI-1 effector	SPA2740	STM2882	7.1	4.5	0.8	1.2
SopE2	SPI-1 effector	SPA1018	STM1855	8.5	4.3	0.6	1.5
SipB	SPI-1 effector	SPA2743	STM2885	17.3	3.1	0.5	1.3
SigD/ SopB	SPI-1 effector	SPA1759	STM1091	7.8	3	0.6	1.5
OrgC	SPI-1 effector	SPA2726	STM2868	10.8	2.3	1.6	2.1
SipD	SPI-1 effector	SPA2741	STM2883	11.2	3.9	0.7	1.2
SteA	SPI-1 effector	SPA1285	STM1583	>>	>>	3	1.4
FliD	hook- associated	SPA0910	STM1960	3.3	3.2	0.7	1.2
FliC	flagellin	SPA0911	STM1959	0	0	0	0
FlgL	hook-associated	SPA1667	STM1184	3.1	3.6	1.6	1.2
FlgK	hook-associated	SPA1668	STM1183	3.1	2.6	1.3	1.2
FlgE	hook- associated	SPA1674	STM1177	2.3	2.2	0.4	0.9
FlgG	basal-body rod protein	SPA1672	STM1179	2.5	2	0.5	0.5
FliE	basal body complex	SPA0902	STM1968	2.1	1.7	1.2	0.9
FlgB	basal-body rod protein	SPA1677	STM1174	6.9	3.9	0.4	0.5
FlgC	basal-body rod protein	SPA1676	STM1175	6.9	3.7	1.6	0.9
FlgM	anti-sigma factor	SPA1679	STM1172	3	2.5	0.4	0.8
FlgN	flagella synthesis	SPA1680	STM1171	2.5	2	ND	1
CheA	chemotaxis protein	SPA0947	STM1960	>>	2.9	1.8	1.6
CheY	chemotaxis protein	SPA0952	STM1916	2.6	2.3	1	1.7
CheW	chemotaxis protein	SPA0948	STM1920	>>	>>	2.5	0.5
FliK	hook-length control	SPA0896	STM1974	1.4	1.4	0.4	0.5
FlgD	basal body rod modification	SPA1675	STM1176	0.9	0.8	0.4	0.3
FlgF	basal body rod protein	SPA1673	STM1178	1.6	1.3	0.2	0.7
FljB	flagellin	*SPA2627*	STM2771	ND	ND	1.5	1.9
FliY	cystine transporter subunit	SPA0915	STM1954	0.6	0.6	1.4	0.3
FlgJ	peptidoglycan hydrolase	SPA1669	STM1182	ND	0.9	ND	1.9
DnaK				0.7	0.8	1	1.2
GroEL				0.7	0.9	0.7	1.4

[a] Shown are the ratios of normalized (label-free quantification) protein intensities between *fliC* and wild-type (WT) backgrounds of *S.* Paratyphi A (SPA) and *S.* Typhimurium (STM) as determined by LC–MS-MS in two independent experiments (exp. 1 and exp. 2). ≫, the protein was not detected (or was detected in very small amounts) in the wild-type strain; ND, not detected.

purified flagellin, restored the ability of the *fliC* or the *flhA* mutant to enter host cells (Fig. 4), demonstrating that functional intact flagella are required for host cell entry. Moreover, since FliC- and FlhA-dependent invasion was apparent even when a mild centrifugal force was applied and considering the similar level of adhesion to that of the wild type, it is possible that the contribution of flagella to host cell invasion is not due to motility deficiency but to other flagellum-mediated functions.

An unexpected regulatory link between flagella and *S.* Paratyphi A SPI-1 was demonstrated when a deletion of *fliC* in *S.* Paratyphi A led to increased expression of the flagellar regulon (classes II and III) and SPI-1 genes (Fig. 5 and 6). Moreover, a quantitative-proteomics approach revealed oversecretion of SPI-1 effectors and flagellar components in the absence of flagellin in *S.* Paratyphi A (Table 2 and Fig. 5C). Ectopic expression of either FliC or FljB counteracted this phenomenon and demonstrated that flagellin can affect the flagellar regulon and SPI-1 on the transcriptional level (Fig. 6).

Flagellar regulation is highly complex and is orchestrated at the levels of transcription, translation, flagellum assembly, and stability, involving a large number of positive and negative regulators, including SirA/BarA (38), RtsB (39), SlyA and LrhA (40), RcsB (41), RflM (42), CsrA (43), FimZ (44), HilD (45), cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex (cAMP-CRC) and H-NS (46), and YdiV (47), as well as other mechanisms. Our results indicate that the lack of flagellin specifically, but not the absence of flagella (as in the *flhA* mutant), results in overexpression of SPI-1 and flagellar genes, including the class II regulator *fliZ*. In turn, FliZ positively regulates SPI-1 genes (Fig. 7). The mechanism by which flagellin absence signals to the flagellar regulon in *S.* Paratyphi A is still unclear and requires a follow-up investigation. This could occur in either a FliC-direct or indirect manner. Although the addition of purified *S.* Typhimurium flagellin to an *S.* Paratyphi A *fliC* mutant culture or subculturing an *S.* Paratyphi A *fliC* strain into the filtered supernatant in a wild-type background (containing a high concentration of secreted FliC monomers) did not change the expression of motility genes (data not shown), we cannot exclude direct regulation by endogenous FliC. An alternative, indirect mechanism could be different responses of *S.* Paratyphi A and *S.* Typhimurium to a disastrous flagellar assembly in the absence of flagellin. For example, if *S.* Paratyphi A still secretes a putative anti-FlhDC factor in

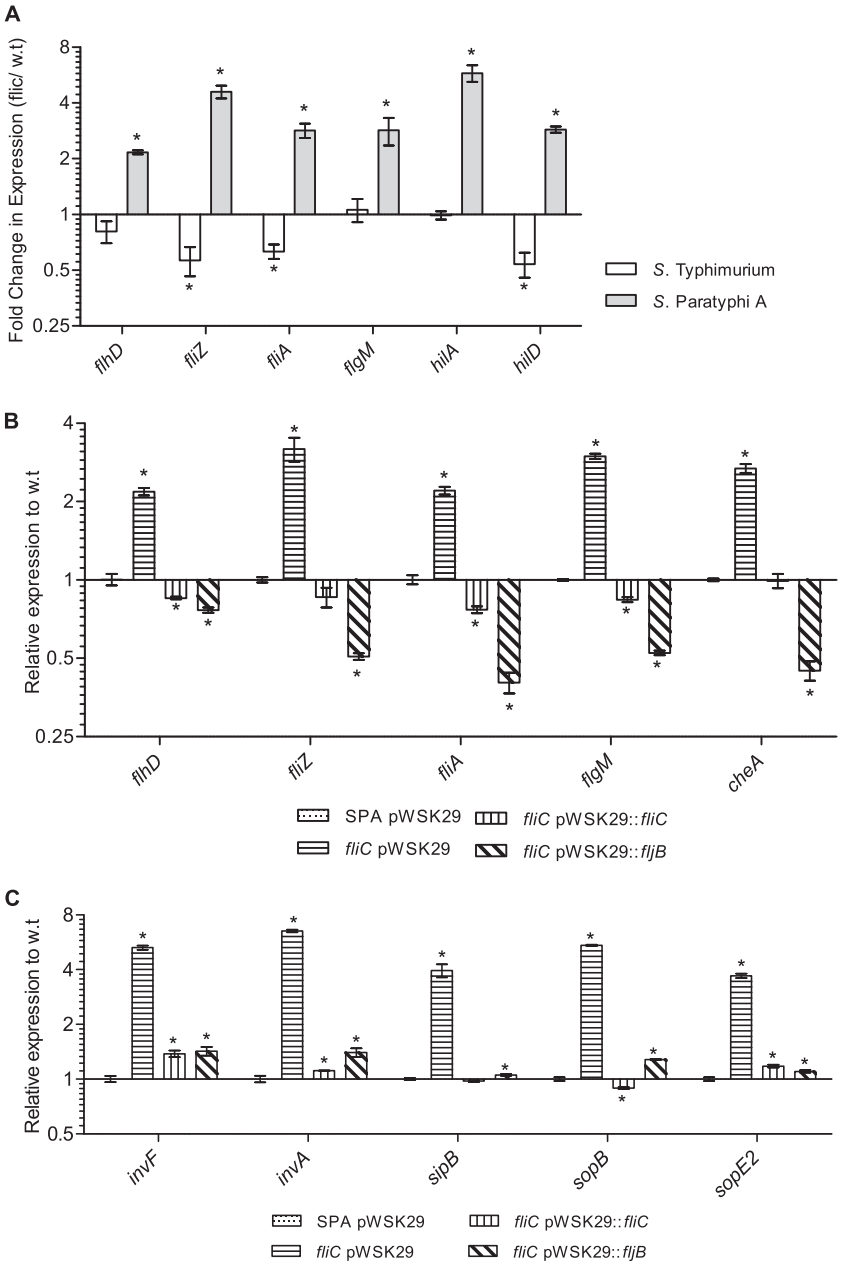

FIG 6 The absence of flagellin affects the transcription of flagellar and SPI-1 genes. (A) Total RNA was harvested from *S.* Typhimurium SL1344 and *S.* Paratyphi A 45157 wild type and *fliC* mutants grown to late logarithmic phase and was subjected to qRT-PCR. The fold changes in the abundances of the *flhD*, *fliZ*, *fliA*, *flgM*, *hilA*, and *hilD* transcripts (normalized to *rpoD*) in the *fliC* strain relative to their expression in the wild-type background are shown. The indicated values represent the means and SEM of the results of three independent qRT-PCR experiments from three independent RNA extractions. (B and C) RNA was extracted from *S.* Paratyphi A 45157 carrying the plasmid pWSK29 and from *S.* Paratyphi A 45157 *fliC*-null mutant strains carrying the vector (pWSK29) or the cloned *fliC* or *fljB* gene. cDNA was subjected to qRT-PCR, and the fold changes in the transcription of the flagellar genes *flhD*, *fliZ*, *fliA*, *flgM*, and *cheA* (B) or the SPI-1 genes *invF*, *invA*, *sipB*, *sopB*, and *sopE2* (C) were normalized to *rpoD*. The fold changes are shown relative to their expression in the wild-type/pWSK29 strain. A two-tailed one-sample *t* test was used to determine statistical significance (*).

the absence of the filament while *S.* Typhimurium blocks its secretion under such conditions, the induction of the motility regulon in the former (Fig. 7A) and its repression in the latter (Fig. 7C and D) can be explained. A previously identified anti-FlhDC factor is FliT (48), which was reported to act as an export chaperone for the filament-capping protein FliD (49). Nonetheless, our efforts to detect FliT in the secreted fractions in the different backgrounds using Western blotting and LC–MS-MS were unsuccessful (data not shown). The involvement of other flagellum-secreted repressors, such as the anti-sigma 28 factor FliM or other, yet uncharacterized regulators, is also conceivable.

Previous studies have shown a regulatory link between the flagellar regulon and SPI-1 via FliZ, which was shown to posttranscriptionally activate HilD, the master regulator that controls SPI-1 expression (28, 29). Coordinated regulation of the flagellar regulon and SPI-1 in a FliZ-dependent manner was also demonstrated to be regulated by

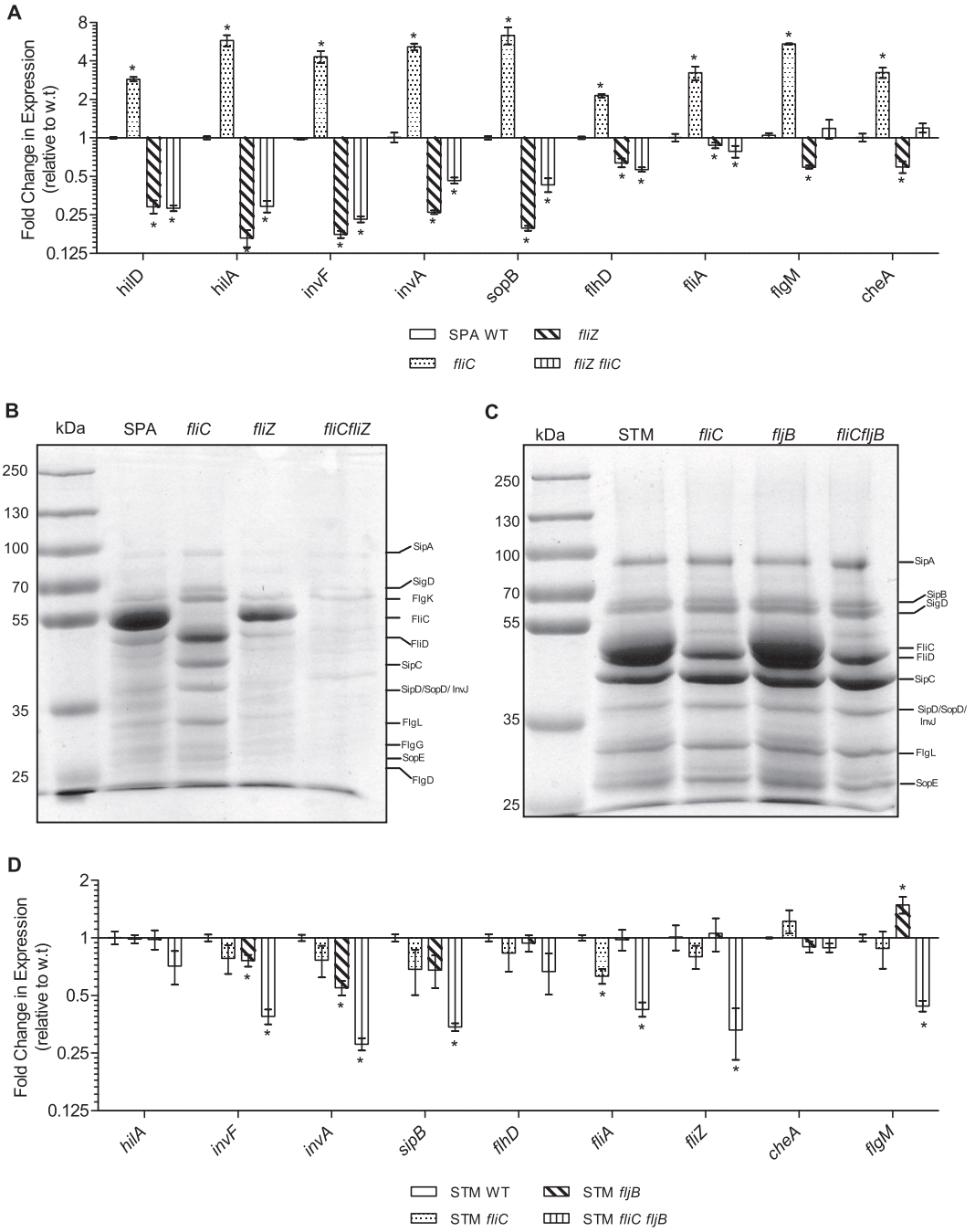

FIG 7 Flagellin absence affects SPI-1 and flagellar gene expression in a FliZ-dependent manner. (A) Total RNA was harvested from *S.* Paratyphi A 45157 wild type and *fliC*, *fliZ*, and *fliC fliZ* mutants grown to late logarithmic phase and was subjected to qRT-PCR. The fold changes in the abundances of the *hilD*, *hilA*, *invF*, *invA*, *sopB*, *flhD*, *fliA*, *flgM*, and *cheA* transcripts (normalized to *rpoD*) were relative to their expression in the wild-type background. The indicated values represent the means and SEM of the results of three independent qRT-PCR experiments from three independent RNA extractions. A two-tailed one-sample *t* test was used to determine statistical significance (*). (B) *S.* Paratyphi A cultures that were grown in LB medium for 5.5 h were normalized to the same optical density, and their supernatant proteins were precipitated by TCA. The precipitated fractions from the wild type (SPA) and *fliC*, *fliZ*, and *fliC fliZ* mutants were separated on SDS-12% polyacrylamide gels and stained with Coomassie G-250. (C) *S.* Typhimurium cultures that were grown in LB medium for 5.5 h were normalized to the same optical density, and their supernatant proteins were precipitated by TCA. Secreted proteins from *S.* Typhimurium wild type (STM) and *fliC*, *fljB*, and *fliC fljB* mutants were separated on SDS-polyacrylamide gels and stained as for panel B. (D) Total RNA was harvested from *S.* Typhimurium SL1344 wild type and *fliC*, *fljB*, and *fliC fljB* mutants grown to late logarithmic phase and was subjected to qRT-PCR. The fold changes in the abundances of the *hilA*, *invF*, *invA*, *sipB*, *flhD*, *fliA*, *fliZ*, *cheA*, and *flgM* transcripts (normalized to *rpoD*) was relative to their expression in the wild-type background. The indicated values represent the means and SEM of the results of three independent qRT-PCR experiments from three independent RNA extractions.

the ClpXP protease, affecting the turnover of the $FlhD_4C_2$ complex, $FlhD_4C_2$ in turn controls FliZ expression, which regulates the cellular levels of HilD (50, 51). Such regulatory cross talk couples the expression of the structurally similar apparatuses of the flagella and T3SS-1 (52) and is expected to coordinate the motility and SPI-1 regulons, which are both required during *Salmonella* invasion (6, 53, 54). Chubiz and colleagues have also shown that FliZ is critical for *Salmonella* colonization and invasion of the intestine but is dispensable during systemic infection (28), demonstrating a tight regulatory and functional linkage between SPI-1 and the flagella *in vivo*, mediated by FliZ.

Interestingly, construction of an *S.* Typhimurium strain that lacks flagellin by an in-frame deletion of both *fliC* and *fljB* did not result in SPI-1 and flagellar gene induction, as in *S.* Paratyphi A (Fig. 7C and D), establishing a unique and previously undescribed regulatory cross talk between flagellin expression and the SPI-1 regulons in *S.* Paratyphi A. Nevertheless, the fact that the absence of FliC decreased host cell entry by both *S.* Typhimurium and *S.* Paratyphi A (Fig. 4) suggests that the impaired invasion is not due to the dysregulation of SPI-1 and is probably related to an independent physiological function of *Salmonella* flagella.

SPI-1 and flagellar gene regulation by flagellin is likely to occur in *S.* Paratyphi A due to genome degradation and, specifically, the lack of FljB, which may alter flagellar assembly or regulation compared to *S.* Typhimurium. Recently, we showed that typhoidal serovars, but not *S.* Typhimurium, respond to fever-like temperature (39 to 42°C) by decreasing their motility and host cell invasion compared to 37°C. We also showed that this phenotype is mediated at the transcriptional level (55). Taken together, our observations reveal novel differences in the regulatory pathways controlling motility and invasion between typhoidal and NTS serovars.

In summary, we showed that typhoidal serovars are less motile than *S.* Typhimurium (Fig. 1) and that this phenotype is associated with lower expression of the entire motility-chemotaxis regulon (Fig. 2). Nevertheless, despite the lower expression, functional flagella were shown to be required for *S.* Paratyphi A invasion of host cells, but not for adhesion (Fig. 3 and 4). In addition, we established, for the first time, different regulatory responses to the lack of flagellin in *S.* Paratyphi A versus *S.* Typhimurium, resulting in overexpression of the flagella and SPI-1 genes and enhanced secretion of SPI-1 effectors in a FliZ-dependent manner that occurs in *S.* Paratyphi A, but not in *S.* Typhimurium (Fig. 5 to 7). We hypothesize that these regulatory differences, the lower expression of the flagellar genes, and the restrained motility affect host-pathogen interactions and contribute to the distinct clinical manifestations resulting from typhoidal versus nontyphoidal infections in humans.

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